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The Effects of Certain Hypocholesterolemic Agents on Hepatic Delta-Aminolevulinic Acid Synthetase Levels in Mice[1] (37826)

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Granick and Urata (1) have shown that δ-aminolevulinic acid synthetase (ALAS) is the rate-limiting enzyme in porphyrin and heme biosynthesis. The enzyme is inducible by certain drugs and steroid metabolites both *in vitro* (2) and *in vivo* (3–5). Evidence has been accumulating which has associated cholesterol metabolism to porphyrin biosynthesis. These findings include (a) the occurrence of concommittant porphyria and hypercholesterolemia in animals treated with porphyrinogenic drugs (6, 7); (b) certain porphyria-producing compounds cause labeled-precursor incorporation into hepatic cholesterol (7); (c) persons with the genetic disease acute intermittent porphyria (AIP) usually present with hypercholesterolemia (8); and (d) persons with AIP have significant increases in beta-lipoprotein (9), the major vehicle of cholesterol transport in the plasma.

In order to further investigate the relationship between cholesterol metabolism and porphyrin biosynthesis, the effect of hypocholesterolemic agents was studied with respect to the rate-limiting enzyme, ALAS. Typically, ALAS activity is inducible to high levels by the porphyrinogenic substances allylisopropylacetamide (AIA) and 3,5-dicarbethoxy-1,4-dihydro-2,4,6-trimethylpyridine (DDC). The hypocholesterolemic compounds used in the present investigation prevented the ability of AIA and DDC to induce ALAS activity to the usual extent.

Materials and Methods. Experimental porphyria. White Swiss Webster female mice, except where indicated, weighing 25–30 g were fed a regular rat chow ration until 24 hr before administration of the porphyrinogenic compounds. Food was then withheld until sacrifice (cervical dislocation), which occurred 12 hr after porphyria-producing agents were administered. Water was available throughout the duration until sacrifice.

Administration of porphyric agents. (a) Allylisopropylacetamide (Hoffman-LaRoche Inc.) was dissolved in 0.90% saline to a final concentration of 20 mg/ml. Three-tenths of a milliliter, corresponding to 6 mg, were injected subcutaneously. (b) 3,5-Dicarbethoxy-1,4-dihydro-2,4,6-trimethylpyridine was synthesized according to the method of Loev and Snader (10). The crude product was purified following the procedure of DeMatteis and Prior (11). Confirmation of the purity of the final product was determined by comparison of the uv and ir spectra with that supplied by Dr. Racz.[3] DDC was dissolved in saline to a final concentration of 30 mg/ml and sonicated for 30 sec (Heat Systems–Ultra-

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sonics Inc.) just before use. Six milligrams were injected subcutaneously 12 hr before sacrifice.

Administration of hypocholesterolemic agents. (a) *Trans*-1,4-Bis-(2-chlorobenzylaminomethyl)-cyclohexane dihydrochloride (AY9944) was obtained from Dr. Dvornik at the Ayerst Research Laboratories. It was dissolved in saline to a final concentration of 15 mg/ml; and 3 mg was injected intraperitoneally daily (24 hr apart) for 3 days. The final injection was made 12 hr before sacrifice. (b) Beta-benzalbutyric acid (BBB) was dissolved in saline to a final concentration of 30 mg/ml; and either 150 mg/kg or 300 mg/kg were injected intraperitoneally daily for 4 days. The compound was obtained from the Instituto Biochimico Italiano, Milan.

Protein determination. Mitochondrial protein was quantitatively determined as described by Cleland and Slater (12) with some modification. To 0.1 cc of mitochondria in 0.25 *M* sucrose–0.1 m*M* EDTA was added 0.1 cc Triton X-100. The mixture was allowed to stand 5 min before addition of 10 ml of 5% trichloroacetic acid (w/v). The precipitate was treated first with 50% ethanol and then 96% ethanol. Upon standing 15 min, the protein was precipitated by centrifugation, and 0.9 ml water plus 4.0 ml biuret reagent (13) was added to the residue. The tubes were mixed with a glass stirring rod and covered. After remaining at ambient temperature overnight, the tubes were read at 540 nm.

Cholesterol determination. Total serum cholesterol was determined by the micromethod of Wybenga *et al.* (14).

Determination of delta-aminolevulinic acid synthetase activity. Liver mitochondria from treated and control mice were obtained essentially as described by Schneider and Hogeboom (15). The washed mitochondria were incubated aerobically for 30–60 min in Dubnoff metabolic shaker at 37°, pH 7.4. The incubation mixture contained 75 m*M* glycine, 10 m*M* potassium succinate, 50 m*M* THAM, 20 m*M* sodium phosphate, 15 m*M* potassium chloride, 5 m*M* magnesium chloride, 2 m*M* EDTA, and 0.1 ml of a 7.5% (w/v) solution of trichloroacetic acid. The deproteinized clear solution was analyzed for aminoketones by the method of Granick (2).

TABLE I. Effect of AY9944 on AIA-Induced Porphyria.

Treatment	No. mice	Specific activity[a]
Saline + Saline	10	2.13 ± 0.44
Saline + AIA	12	7.61 ± 0.47
AY9944 + AIA	26	1.65 ± 0.20
AY9944 + saline	16	1.69 ± 0.09

[a] Expressed as nanomoles of ALA formed per hour per milligram of mitochondrial protein. Values are averages of three different experiments, each in duplicate, plus or minus the SEM.

Results. Table I shows the effect of a hypocholesterolemic agent, AY9944, on ALAS activity. This compound inhibits the conversion of 7-dehydrocholesterol to cholesterol (16). The data show the results of experiments in which mice were given three prior ip injections of AY9944 or control solvent daily plus the inducer drug AIA (one time). The hypocholesterolemic agent prevented the usual induction of ALAS activity by AIA. In fact, the level of enzymatic activity was slightly lower in the mice injected with AY-9944 plus saline than the saline-injected controls. Table II shows that this same agent prevented the experimental porphyria usually produced by another inducer substance, DDC. However, the effect of AY9944 with AIA was more pronounced, i.e., AIA was more effective in producing experimental porphyria than DDC, and the inhibition by AY-9944 was more pronounced when AIA was subsequently administered.

TABLE II. Effect of AY9944 on DDC-Induced Porphyria.

Treatment	No. mice	Specific activity[a]
Saline + DDC	18	4.87 ± 0.19
AY9944 + DDC	20	2.53 + 0.33

[a] Expressed as nanomoles of ALA formed per 30 minutes per milligram of mitochondrial protein. Values are averages of two different experiments, each in duplicate, plus or minus the SEM.

TABLE III. Serum Cholesterol Levels.

Treatment	No. mice	Serum cholesterol[a] (mg/dl)
Saline + saline	6	90.9 ± 1.8
Saline + AIA	8	156.6 ± 2.4
Saline + DDC	6	174.5 ± 2.8
AY9944 + saline	6	79.6 ± 2.1
AY9944 + AIA	6	121.0 ± 4.7
AY9944 + DDC	6	154.0 ± 3.2

[a] Values are averages of four determinations, each in duplicate, plus or minus the SEM.

In order to determine whether the interference with δ-aminolevulinic acid (ALA) production was associated with the hypocholesterolemic agent actually lowering cholesterol levels, total serum cholesterol was measured. Table III shows the serum cholesterol content under experimental conditions identical to those used to prevent hepatic porphyria in mice. The three injections of AY9944 which prevent induction of hepatic ALAS activity by AIA (Table I) substantially lower the serum cholesterol level in the AIA-treated mice by more than 20%. The serum cholesterol concentration in the saline-treated and DDC-treated mice, however, was lowered approximately only 10%.

Another hypocholesterolemic agent, β-benzalbutyric acid (BBB), was tested also in combination with AIA. It showed a similar ability to prevent full expression of experimental porphyria in female mice (Table IV). However, this agent had a much smaller effect in male mice, all other factors being kept constant. An explanation of this apparent sex difference in the response is not presently available.

TABLE IV. Effect of Beta-Benzalbutyric on Hepatic ALAS Activity.

Treatment	Sex	No. mice	Specific activity[a]
Saline (control)	F	10	2.13 ± 0.44
Saline + AIA	F	4	6.78 ± 0.07
β-Benzalbutyrate + AIA	F	8	3.54 ± 0.24
Saline + AIA	M	12	5.64 ± 0.25
β-Benzalbutyrate + AIA	M	18	4.37 ± 0.34

[a] Expressed as nanomoles of ALA formed per hour per milligram of mitochondrial protein. Values are averages of two different experiments, each in duplicate, plus or minus the SEM.

Discussion. Granick and his coworkers (2) discovered that not only exogenously administered compounds, but also endogenously produced substances, i.e., steroids, were capable of stimulating porphyrin synthesis. Subsequent studies demonstrated that the type of steroids responsible for porphyrin induction via ALAS, the rate-limiting enzyme in heme biosynthesis, were the 5β-H steroids, the reduced metabolites of the primary hormones. Goldberg *et al.* (17) found increases in urinary steroid metabolites (conjugated) in patients with AIP. Recently, Kappas *et al.* (18, 19) have attributed the increase in 5β-H steroids in these patients to a defect in Δ4-5α reductase activity. As a result, more 5β-metabolites presumably become available for the induction of ALAS.

Injection of four compounds which produce hepatic porphyria in mice increased plasma cholesterol significantly (7). Labeled-precursor incorporation into hepatic cholesterol occurred with three of the drugs. However, DDC was an exception. These authors suggest that these drugs may play a role in the stimulation of cholesterol anabolism or in the inhibition of cholesterol catabolism in experimental porphyria resulting in hypercholesterolemia.

Although the present experiments support the evidence that porphyrinogenic substances produce a hypercholesterolemia, only AIA induction of ALAS activity was fully prevented by the hypocholesterolemic agents employed (AY9944 and BBB). It is noteworthy that inhibition of hypercholesterolemia was greater than 20%. However, the porphyrinogenic effect of DDC, which also induces the enzyme to high levels, was only partially prevented. This may be related to its less effective response to the cholesterol-lowering agent, AY9944.

It would seem appropriate to attempt to relate the increases in serum cholesterol levels as a source of potential steroid metabolite inducers for ALAS. In fact, this may in part be the case in AIA induction

of ALAS in which AY9944 lowered cholesterol levels and prevented ALAS induction. Thus, the prevention of experimental porphyria may reside at least in part in the capability of these hypocholesterolemic agents to lower serum cholesterol levels. Further studies will be needed to more fully clarify these points.

Summary. The effects of the hypocholesterolemic agents, *trans*-1,4-bis-(2-chlorobenzylaminomethyl)-cyclohexane dihydrochloride (AY9944) and beta-benzalbutyric acid (BBB), on the level (activity) of delta-aminolevulinic acid synthetase (ALAS) in the mitochondria of livers of mice injected with certain porphyrinogenic compounds were studied. The porphyrinogenic agents employed were allylisopropylacetamide (AIA) and 3,5-dicarbethoxy-1,4-dihydro-2,4,6-trimethylpyridine (DDC). All substances were administered parenterally.

Both AY-9944 and BBB injections prevented the increase in hepatic mitochondrial ALA-synthetase levels which otherwise follow the injection of AIA or DDC. AY-9944 administration also produced a consistent decrease in serum cholesterol levels. These data suggest that a decrease in the hepatic steroid "pool" and presumably that of 5-beta-H-steroid metabolites, as reflected by the serum cholesterol level, is associated with the observed decrease in hepatic mitochondrial ALAS.

The present data thus support, by an entirely different approach, other evidence implicating steroid metabolites in the regulation of porphyrin–heme biosynthesis in a mammalian species.

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